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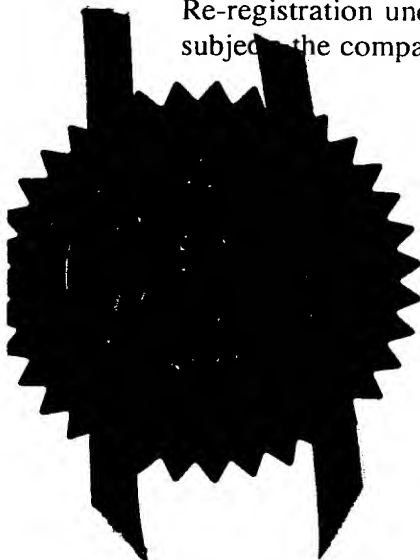
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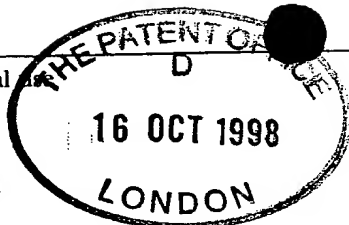
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19OCT98 E398197-4 052029
P01/7700 0.00 - 9822714.3

Your Reference:

B45160

16 OCT 1998

9822714.3

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**The
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Request for grant of a

Patent

Form 1/77

Patents Act 1977

① Title of invention

1 Please give the title of the invention

VACCINES

②

Applicant's details

☐

First or only applicant

2a

If you are applying as a corporate body please give:
Corporate Name

SmithKline Beecham

11/11 14
15/12/97
Biologicals
sq.

Country (and State of incorporation, if appropriate)

Belgium

2b

If you are applying as an individual or one of a partnership please give in full:

Surname

Forenames

2c

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7533243001
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Filing Date
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Continuation sheets for this Patents Form 1/77

—

Claim(s) Description 15

Abstract Drawing(s)

8b Which of the following documents also accompanies the application?

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Patents Form 7/77 - Statement of Inventorship and Right to Grant

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Patents Form 9/77 - Preliminary Examination Report

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Patents Form 10/77 - Request for Substantive Examination

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⑨ Request

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Vaccines

The present invention relates to new vaccine compositions, processes for their manufacture and their use in medicine. In particular, the present invention relates to improved Hepatitis A vaccines adjuvanted with a potent immunostimulator, preferably such as monophosphoryl lipid A or derivative thereof. The invention also relates to combination vaccines in which the Hepatitis A vaccine is a component.

Hepatitis A vaccines are known. For example the vaccine Havrix (Trade Mark), from SmithKline Beecham Biologicals can be used to prevent hepatitis A infections and is also formulated with aluminium hydroxide as adjuvant. This vaccine is produced according to a modified procedure (no CS CL gradient) of Andre et al. It comprises an attenuated strain of the HM-175 Hepatitis A virus inactivated with formol (formaldehyde); see Andre et al [Prog Med. Virol. 1990, vol 37; -p72-95]. The vaccine Twinrix (Trade Mark) which is a combination of the above hepatitis A and hepatitis B antigens may be used to protect against Hepatitis A and Hepatitis B simultaneously.

International patent application WO93/19780 (SmithKline Beecham Biologicals s.a.) discloses, inter alia, a Hepatitis A vaccine adjuvanted with 3D-MPL.

European patent 0 339 667 (Chemo Sero) describes the general concept of combining a hepatitis A antigen and a hepatitis B antigen to make a combination vaccine. In that specification it is stated that the adjuvant which is used is not critical: it must only be capable of enhancing the immune activity to a desired extent and not cause any side effects. It is stated that aluminium gel may be used, in particular aluminium hydroxide gel and aluminium phosphate gel.

It has now been found that traditional processes for producing and purifying inactivated virus for hepatitis A vaccines leave a small residue of contaminants from the host cells upon which the hepatitis A vaccine was grown. Such host cell contaminants are at a level that provide no concern when the vaccine is adjuvanted with aluminium salts, but when adjuvanted with strong immunostimulants it is possible that a vaccinee may raise an adverse immune response to the host contaminants.

Accordingly there is a need for a method of manufacture which removes substantially all traces of such host cell proteins.

Accordingly in one embodiment of the invention there is a process for the production of inactivated Hepatitis A virus substantially free of host cell

5 contamination, the process comprising:

a) a culturing Hepatitis A vaccine and harvesting said hepatitis A preparation

b) treating said hepatitis A preparation with a protease, thereafter

c) separating intact virus from small protease digested protein

10 d) inactivating said virus.

Preferably the Hepatitis A virus is derived from HM-175 strain.

By substantially free of host cell contamination it means that less than 10%, preferably less than 8% more preferably less than 5% protein can be detected by method SDS PAGE and silver staining. As determined by slot blot method one
15 dose of HAV in the vaccine preferably contains less than 10 ng of host cell proteins.

Preferably the protease used is trypsin. Other proteases that may be utilised included pronase, papain, and pepsin.

The protease treatment is preferably carried out at above room temperature,
20 e.g. at about 37°C for about 2 hrs.

The separation of the intact virus from the protease and the digested components can be achieved by permeation chromatography.

Alternatively the protease may be separated by any chromalographic method that separates on the basis of size, for example ultra filtration.

25 The product can then be further purified, by other steps to remove other contaminants. For example by subjecting the product to ion-exchange chromatography to remove any nucleic acid contamination.

Accordingly, in one embodiment of the present invention there is provided an inactivated Hepatitis A virus substantially free of contaminating host proteins.

30 The inactivated hepatitis A virus vaccine may then be formulated into a vaccine. Thus the invention provides a Hepatitis A vaccine comprising an inactivated hepatitis A virus substantially free of host cell contaminants.

Such a vaccine may advantageously include a suitable adjuvant. Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or
5 anionically derivatised polysaccharides, or polyphosphazenes.

Advantageously, the highly purified hepatitis A virus may be formulated with strong adjuvant systems. Thus in the formulation of the invention, it is preferred that the adjuvant composition induces an immune response comprising TH1 aspects. Suitable adjuvant systems include, for example a combination of
10 monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salts.

An enhanced system involves the combination of monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is
15 quenched with cholesterol as disclosed in WO 96/33739.

A particularly potent adjuvant formulation involving QS21 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210.

Other known adjuvants which may be included are CpG containing oligonucleotides.

20 Accordingly in a preferred embodiment of the present invention there is provided a vaccine comprising a virus of the present invention, adjuvanted with a monophosphoryl lipid A or derivative thereof.

Preferably the vaccine additionally comprises a saponin, more preferably QS21.

25 Preferably the formulation additionally comprises an oil in water emulsion and tocopherol. The present invention also provides a method for producing a vaccine formulation comprising mixing a protein of the present invention together with a pharmaceutically acceptable excipient, such as 3D-MPL.

The purified virus of the invention may advantageously be combined with
30 other antigens so that it is effective in the prophylaxis or treatment of other diseases in addition to hepatitis A infections. A preferred combination involves a combination containing a hepatitis B antigen.

The preparation of Hepatitis B surface antigen (HBsAg) is well documented. See, for example, Harford et al in **Develop. Biol. Standard** 54, page 125 (1983), Gregg et al in **Biotechnology**, 5, page 479 (1987), EP-A-0 226 846, EP-A-0 299 108 and references therein.

5 As used herein the expression 'Hepatitis B surface antigen' or 'HBsAg' includes any HBsAg antigen or fragment thereof displaying the antigenicity of HBV surface antigen. It will be understood that in addition to the 226 amino acid sequence of the HBsAg S antigen (see Tiollais et al, *Nature*, 317, 489 (1985) and references therein) HBsAg as herein described may, if desired, contain all or part of
10 a pre-S sequence as described in the above references and in EP-A- 0 278 940. In particular the HBsAg may comprise a polypeptide comprising an amino acid sequence comprising residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L-protein of HBsAg relative to the open reading frame on a Hepatitis B virus of ad serotype (this polypeptide is referred to as L*; see EP 0 414
15 374). HBsAg within the scope of the invention may also include the preS1-preS2-S polypeptide described in EP 0 198 474 (Endotronics) or analogues thereof such as those described in EP 0 304 578 (McCormick and Jones). HBsAg as herein described can also refer to mutants, for example the 'escape mutant' described in WO 91/14703 or European Patent Application Publication Number 0 511 855 A1,
20 especially HBsAg wherein the amino acid substitution at position 145 is to arginine from glycine.

Normally the HBsAg will be in particle form. The particles may comprise for example S protein alone or may be composite particles, for example (L*,S) where L* is as defined above and S denotes the S-protein of HBsAg. The said
25 particles is advantageously in the form in which it is expressed in yeast.

The invention in a further aspect provides a vaccine formulation as described herein for use in medical therapy, particularly for use in the treatment of prophylaxis of hepatitis viral infections. In a preferred aspect the vaccine accordingly to the invention is a therapeutic vaccine useful for the treatment of
30 ongoing hepatitis infections, more especially hepatitis A and/or hepatitis B infections in humans suffering therefrom.

In view of the surprisingly efficacious results obtained, in a further preferred aspect the invention provides a vaccine composition for the treatment of prophylaxis of Hepatitis A and/or Hepatitis B infections.

Advantageously the hepatitis vaccine composition of the invention contains
5 other antigens so that it is effective in the treatment or prophylaxis of one or more other bacterial, viral or fungal infections.

Accordingly the hepatitis vaccine formulation according to the invention preferably contains at least one other component selected from non-hepatitis antigens which are known in the art to afford protection against one or more of the
10 following diseases:

diphtheria, tetanus, pertussis, Haemophilus influenzae b (Hib), and polio.

Preferably the vaccine according to the invention includes HBsAg as hereinabove defined.

Suitable components for use in such vaccines are already commercially
15 available and details may be obtained from the World Health Organization. For example the IPV component may be the Salk inactivated polio vaccine. The pertussis vaccine may comprise whole cell or acellular product.

Advantageously the hepatitis or combination vaccine according to the invention is a paediatric vaccine.

20 The amount of antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending on which specific immunogens are employed. Generally it is expected that each dose will comprise 0.01 to 1.0 μ g protein/dose immunogen, most preferably from between .06 to .150 μ gprot/dose.

25 An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects may receive a boost in about 4 weeks.

In a further aspect of the present invention there is provided a method of manufacture of a vaccine effective in preventing or treating hepatitis infection,
30 wherein the method comprises mixing the hepatitis antigen as defined herein with MPL or derivative thereof.

Using this method one or more additional components are preferably admixed with the inactivated Hepatitis A vaccine to provide a combination vaccine.

The following examples illustrate the invention and its advantages.

Examples:

- 5 Purification of Hepatitis A virus strain HM-175 was carried out according to the protocol shown in Figure 1.

Hepatitis A virus HM-175 was cultured on MRC5 cells (Andre et al supra) and the virus harvested in the presence of detergent - Tween- in order to lyse the cells and then after washing to remove serum contamination, filtered through a
10 0.22µm membrane and subjected to ultra filtration followed by gel permeation chromatography on a sepharose 4B-CL-FF prior to treatment with trypsin.

TRYPSINATION

The pool of fractions containing the HAV virus is treated with purified trypsin extracted from pig pancreas. The trypsin used is double crystallised and
15 kept frozen before use. Before addition of trypsin the pool is prewarmed at 37°C under constant agitation. Trypsin is then added with a ratio of 440 IU per ml of pool, and the mixture is gently stirred for minimum 2H at 37°C (maximum 2.5H).

CONCENTRATION

After trypsin treatment the product is processed without delay at ambient
20 temperatures on an ultrafiltration device in order to reduce the volume. The membrane used is regenerated cellulose with nominal cut-off of 30.000 Dalton, and up to maximum 8ml of trypsinated product per cm² of membrane, is processed at a transmembrane pressure: between 0.2 and 0.6 bar to achieve a concentration factor of between 8 and 12.

25 2nd PERMEATION CHROMATOGRAPHY

The aim of this step is to separate trypsin digested proteins from the unaffected HAV viruses. The separation gel used is Permeation Sepharose 4BFF. The virus is eluted at a smaller retention volume than the smaller protein fragments which are eluted with larger retention volume (closer to the total volume of the
30 column).

Chromatography parameters are as follow:

Chromatographic medium: Sepharose 4B FF (from Pharmacia)

Injected volume: 1 to 5% of gel volume

Elution rate: 5 cm/h

Temperature: 10 to 16°C.

Pool of fractions: target 100 ng prot/ 720 Elisa units (+/- 10%)

5 ION EXCHANGE

The purpose of this purification step is to reduce the DNA content (originating from MRC5 cells).

This step is run according to the batch principle.

10 The pool from the previous chromatographic step is adjusted to 0.3M NaCl and then mixed with the ion exchange resin under mild agitation for 1H (maximum 1.5H) at room temperature.

After DNA fixation the gel is eliminated by filtration.

The unfixed HAV viruses suspension is then diluted to adjust the NaCl concentration to 150mM.

15 The final purified product is sterile filtered on 0.22µm filter.

Chromatographic parameters are as follow:

Load: 3% of gel compared to the volume of the pool (vol/vol).

Temperature: ambient

20 Inactivation was carried out as described in Andre et al, except that 100µg/ml of phenol was used. 250 µg/ml formol

FORMALDEHYDE REDUCTION

Within 48H after the end of the inactivation the product is diafiltered and concentrated in order to reduce the formaldehyde content and to be preadsorbed on aluminium hydroxide.

25 Before use the complete ultrafiltration device is sanitised with 0.1N NaOH for at least 30 minutes. The device is then thoroughly rinsed with diafiltration buffer and the membranes are then coated with a buffer containing amino acids (Travasol).

Finally the device is rinsed with diafiltration buffer.

30 After diafiltration and concentration the final product is sterile filtered on 0.22µm filter.

Example 1b

In an alternative purification scheme, the trypsinisation step is carried out between the ultrafiltration and first permeation chromatography steps. This means that the second permeation chromatography step can be eliminated leading to higher yields of the final product.

Example 2**Characterisation:**

Samples of purified product was analysed by SDS PAGE 12.5 % acrylamide, 1% SDS in the stacking gel, migration for 15 h at 45-50 volts. Gel is stained with AgNO₃ and the colour is allowed to develop for 10 to 20 min and compared with traditional HAV processes (Andre et al).

As can be seen from figure 3 and 4 subjecting the product to protease treatment a majority of high molecular weight contaminants are removed

Example 3**15 HAV vaccine formulations****3.1 HAV - alum 3D-MPL**

The HAV particle of example 1 is first adsorbed on to aluminium hydroxide (superfos) followed by the addition of free 3D-MPL. A 0.5 ml dose 720 ELU of Hepatitis A virus particle / 0,025 mg Al³⁺ ion and 50µg of 3D-MPL.

3.2 HAV + Hbs Ag formulations

The following formulations were made:

1. Hep B S Ag 20µg / AlPO₄ + HA 720 / Al(OH)₃
2. Hep B S Ag 20µg / AlPO₄ / 3D-MPL 50 µg + HA 1440 / Al(OH)₃
- 25 3. Hep B S Ag 20µg / AlPO₄ + 3D-MPL 50 Mg / Al (OH)₃ + HA 720 / Al(OH)₃
4. Hep B S Ag 20µg / AlPO₄ + 3D-MPL 50µg / AlPO₄ + HA 720 Al(OH)₃
5. Hep B S Ag 20/µg / AlPO₄ / 3D-MPL 50µg + HA 720 / Al(OH)₃

30 In group 1 the individual antigens are adsorbed on to the aluminium salt 0.025mg Al³⁺ ion (Al₃OH Superfos) for HA, 0.475 mg Al³⁺ ion (AlPO₄ Superfos type). In group 2 and 5, 50µg/dose of free 3D-MPL is added to

adsorbed Hepatitis S antigen to which the adsorbed hepatitis A component is added. In group 3 and 4, 3D-MPL is separately adsorbed on to the aluminium salt, and then the three adsorbed components are mixed together.

Example 4 - Immunogenicity experiments

5 Balb/c mice

Groups of 10 mice were immunised intramuscularly three times at 2 weeks interval with HAV/HBs formulations (1/10 HD). Antibody response to Hbs were monitored by ELISA at 14 days post II and 14 days post III. The isotypic profile of the anti-HBs response was analysed at 14 days post II. Antibody response to HAV was monitored 14 days post III.

NMRI mice

Groups of 10 mice were immunised intraperitoneally once with HAV/HBs formulations (1/2 HD). Antibody response to Hbs and HAV were monitored by ELISA at 28 days post injection.

15 Formulations

Group	Vaccine lot	Formulation
1	HAB112B6	HBs 20µg / AlPO ₄ + HAV 720 / Al(OH) ₃
2	DHAB713	HBs 20µg / AlPO ₄ / MPL 50 + HAV 1440 / Al(OH) ₃
3	DHAB717	HBs 20µg / AlPO ₄ + MPL 50 / Al(OH) ₃ + HAV 720 / Al(OH) ₃
4	DHAB718	HBs 20µg / AlPO ₄ + MPL 50 / AlPO ₄ + HAV 720 / Al(OH) ₃
5	DHAB716	HBs 20µg / AlPO ₄ / MPL 50 + HAV 720 / Al (OH) 3

HAV Mouse Serology

Quantitation of anti-Hepatitis A Virus antigen (HAV) antibody was performed using Enzygnost kit from Behring (ref: OQEC11). This assay is an ELISA based on the competitive test principle, run in one step and initially developed for human serology.

Two-fold dilution of mice sera (4 dilutions starting at 1/10) human anti-HAV reference (8 dilutions starting at 80 mIU/ml) and controls were performed in anti-HAV negative human sera. Mixtures of test/control samples (25µl), HAV

antigen solution (50µl) and anti-HAV mouse monoclonal conjugated with
peroxydase (50µl of 1/41 dilution performed in conjugate buffer) were incubated on
HAV pre-coated microplates for 2 hrs at 37°C. The plates were then washed and
incubated for 30 min with a solution of TMB (100µl). The reaction was stopped
5 with H₂SO₄ 0.5N and read at 450/620 nm.

Anti-HAV antibody titers were calculated from the reference by SoftmaxPro
(using a four parameters equation) and expressed in mIU/ml.

Results

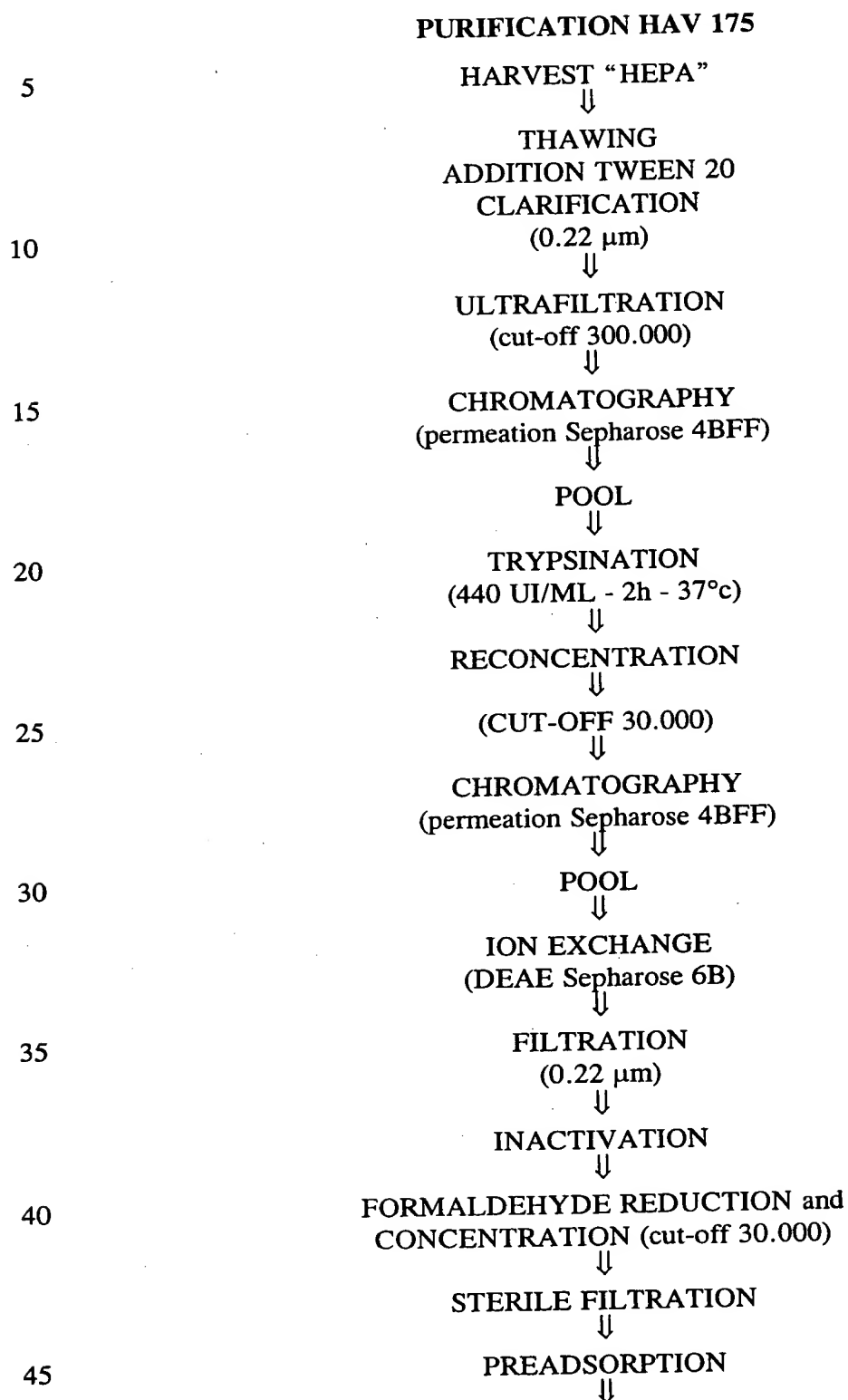
The results are shown in figure 2.

10

In figure 2a results demonstrate that formulations containing MPL induce
significantly higher antibody responses to the hepatitis A component than the
aluminum salt group alone. Similarly the results shown in figure 2b demonstrate
that MPL containing formulations induce higher antibody titres to HbsAg.

15

Figure 1



B45160

STORAGE
↓
FORMULATION
↓
FILLING

5

Figure2a

PROJECT A+B/MPL / EXP 98299 : Serological analysis

Mice: NMRI	Antigen: HB, HA	Vehicule: Alum
Route: sc	Immunostimulant: MPL	
Immunisation scheme (day): 0,28		

1	HAB112B6	HB20/ AIPO4 + HA720/ Al(OH)3
2	DHAB713	HB20/AIPO4 / MPL50 + HA1440/ Al(OH)3
3	DHAB717	HB20/ AIPO4 + MPL50/Al(OH)3 + HA720/ Al(OH)3
4	DHAB718	HB20/ AIPO4 + MPL50/AIPO4 + HA720/ Al(OH)3
5	DHAB716	HB20/AIPO4 / MPL50 + HA720/ Al(OH)3

1) Response on pool on 28 days post I

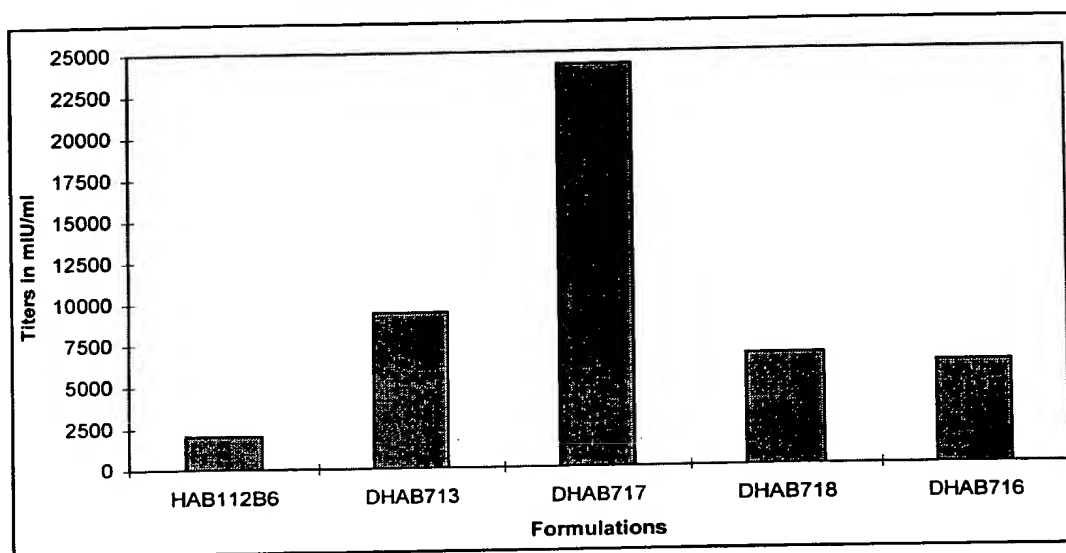


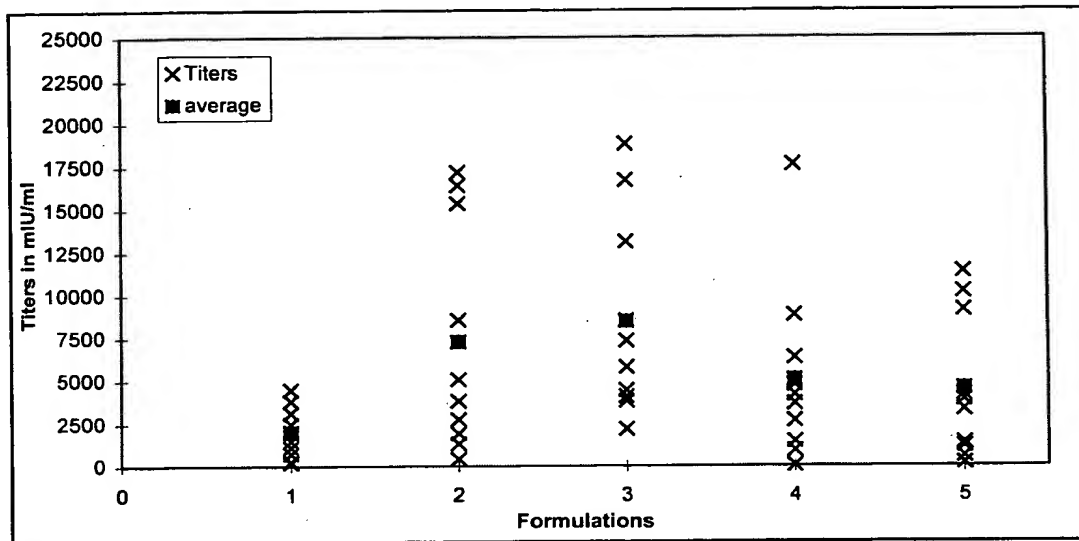
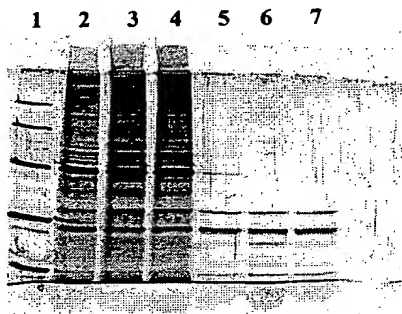
Figure 2b**2) Response on individuals on 28 days post I**

Figure 3

SDS PAGE of HAV pool before utilising Andre et al PROCESS) & after the MMP process of example 1b (HAV-MMP) Early trypsin single permeation step process.
 Proteins are fractionated on 12.5% SDS-PAGE. They are visualized by silver staining

Lane 1: Low MW standards (14.4,21.5,31.0,45.0,66.2,97.4 kDa)
 Lane 2: Andre et al (lot 5199)
 Lane 3: Andre et al (lot 5200)
 Lane 4: Andre et al (lot 5201)
 Lane 5: HAV-MMP (lot 7199)
 Lane 6: HAV-MMP (lot 7200)
 Lane 7: HAV-MMP (lot 7201)

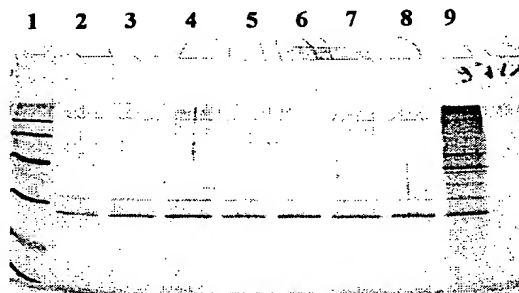


Note: lot numbers refer to distinct virus pools

5 Figure 4

SDS PAGE of HAV pool before (andre et al) & after the process of example 1a with two step permeation chromatography (HAV-MMP)
 Proteins are fractionated on 12.5% SDS-PAGE. They are visualized by silver staining

Lane 1: Low MW standards (14.4,21.5,31.0,45.0,66.2,97.4 kDa)
 Lanes 2 to 8: HAV-MMP (lots 6029 to 6032 and 6034 to 6036)
 Lane 9: Andre et al (lot 5010)



Note: - lot numbers refer to distinct virus pools
 -Scan of a gel photograph

